Spliceosome-Mediated *Trans*-Splicing: The Therapeutic Cut and Paste

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Spliceosome-mediated RNA *trans*-splicing (SMaRT) is an RNA-based technology to reprogram genes for diagnostic and therapeutic purposes. For the correction of genetic diseases, SMaRT offers several advantages over traditional gene-replacement strategies. SMaRT protocols have recently been used for *in vitro* phenotypic correction of a variety of genetic disorders, ranging from epidermolysis bullosa to neurodegenerative diseases. *In vivo* studies are currently bringing *trans*-splicing RNA therapy toward clinical application. In this review, we summarize the progress made toward the medical use of SMaRT and provide an outlook on its upcoming applications.

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INTRODUCTION

The long-held view in molecular biology of RNA fulfilling a role solely as an intermediate product between DNA and protein has been revised and expanded by the discovery of important regulatory functions of RNAs. Thus, different classes of messenger RNA (mRNA), transfer RNA, and also non-coding regulatory RNAs such as micro RNA and short hairpin RNA have been studied intensively in gene regulation, epigenetics, and for therapeutic purposes (Morris, 2008).

Gene reprogramming at the mRNA level offers a wide range of novel therapeutic applications in medicine. Possible areas of use that have been documented include correction of genetic diseases (Wally *et al.*, 2010; Murauer *et al.*, 2011), enhancement of therapeutic modalities in cancer medicine (Gruber *et al.*, 2011), molecular imaging of gene expression (Walls *et al.*, 2008), mRNA knockdown (Siomi and Siomi, 2009), and therapeutic protein production (Iwasaki *et al.*, 2009; Wang *et al.*, 2009).

For the correction of pathogenic mutations in genetic diseases, somatic gene therapy has been studied thoroughly. This involves supplementation of the mutated gene in target cells with its wild-type complementary DNA (cDNA) copy to obtain clinical benefit (Friedmann and Roblin, 1972). Although cDNA therapy has been brought to clinical application for numerous genetic diseases, including ß-thalassemia (Perumbeti and Malik, 2010), junctional epidermolysis bullosa (Mavilio *et al.*, 2006), and primary immunodeficiencies (Aiuti and Roncarolo, 2009) among others, this gene-addition strategy has limitations: (a) large genes exceed the packaging capacities of commonly used viral vectors; (b) transgene expression can only be regulated by inclusion of endogenous promoters into the construct; (c) in autosomal dominant diseases caused by heterozygous missense mutations, the mutant allele (which remains intact within the cell) may continue to exert its negative effect; and (d) overexpression of the transgene driven by strong viral promoters can lead to deleterious effects on the target cell.

These technological bottlenecks of gene therapy can be circumvented by spliceosome-mediated RNA *trans*-splicing (SMaRT). First, the natural expression pattern of the target gene is retained, as *trans*-splicing can take place only in cells where the target pre-mRNA is expressed, thereby minimizing ectopic expression spatially, temporally, and quantitatively. Second, the segmental introduction of therapeutic nucleic acid sequences allows much smaller correcting molecules to be used, circumventing packaging limitations. Third, *trans*-splicing converts the mutant allele to a wild-type allele, thereby not only knocking down the expression of the dominant-negative allele but also increasing the level of wild-type protein by simply ablating the mutant form.

MODES OF *TRANS*-SPLICING

Currently, two distinct modes of *trans*-splicing-based RNA reprogramming are being studied. SMaRT exploits the cell's endogenous spliceosome to *trans*-splice an exonic region, delivered by an RNA-*trans*-splicing molecule (RTM), into an endogenous target pre-mRNA. Thereby, a 5′-, 3′-, or internal portion of a mutated target mRNA can

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Abbreviations: BD, binding domain; CD40L, CD40 ligand; CFTR*, cystic fibrosis transmembrane conductance regulator; IER, internal exon replacement; mRNA, messenger RNA; RDEB, recessive dystrophic epidermolysis bullosa; RTM, RNA-*trans*-splicing molecule; SCC, squamous cell carcinoma; SMA, spinal muscular atrophy; SMaRT, spliceosome-mediated RNA* trans*-splicing*

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be replaced. The second method uses an autocatalytic group I intron ribozyme designed to bind and *trans*-splice therapeutic RNA sequences onto a target transcript, referred to as ribozyme-mediated RNA *trans*-splicing (Sullenger and Cech, 1994).

In contrast to SMaRT, ribozyme *trans*-splicing is limited to the replacement of the 3′-part of a transcript. Several *in vitro* and *in vivo* studies have demonstrated the ability of *trans*-splicing ribozymes to repair defects in a variety of human diseases, such as sickle cell anemia (Byun *et al.*, 2003), ovarian cancer (Shin *et al.*, 2004), and pancreatic cancer (Kastanos *et al.*, 2004; Shin *et al.*, 2004). Remaining issues in ribozyme technology include the fact that the reactions are reversible and that the majority of the endogenous target sequences are inaccessible to the ribozymes because of complex structural folding and interactions with a great number of proteins *in vivo*. In addition, ribozyme RNA is relatively expensive to synthesize (for a review see Fiskaa and Birgisdottir, 2010).

Thus, we focus on SMaRT. Here, we summarize the current ''state of the art'' and recent progress in the therapeutic use of SMaRT technology, and give an outlook on its upcoming clinical applications.

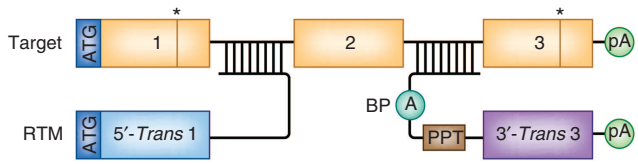

Figure 1. Schematic depiction of RNA-*trans*-splicing molecules (RTMs). Whereas a 5′-RTM consists of the respective 5′-gene portion to be replaced (5′-*trans1*), as well as a binding domain specifically hybridizing to the targeted intron, 3′-RTMs (3′-*trans3*) also have to contain a branch point (BP) and a poly pyrimidine tract (PPT). *Mutation to be replaced.

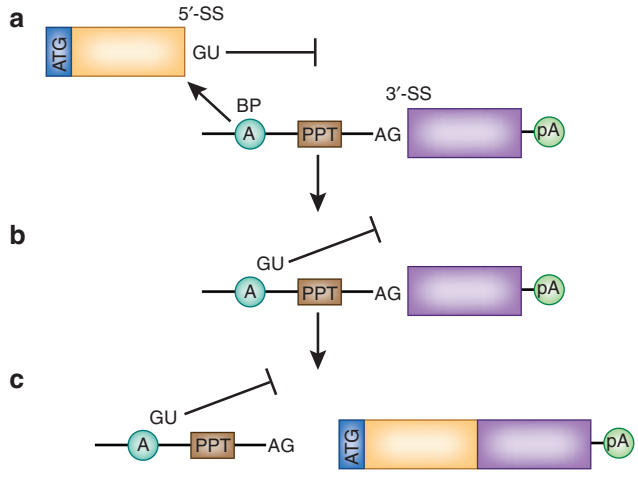

Figure 2. *Trans*-splicing occurs through two *trans*-esterification reactions. (**a**) The 2′-OH group from the adenosine residue of the branch point (BP) on the RNA-*trans*-splicing molecule (RTM) attacks the phosphoryl group at the 5′-splice site (5′-SS) at the target pre-mRNA. (**b**) As a result, the 5′-exon is released and the 5′-end of the RTM forms a Y-branched intermediate. Further, the 3′-OH of the 5′-exon attacks the phosphoryl group at the 3′-SS, (**c**) resulting in displacement of the 3′-end of the target and joining of the 5′-target exon with the RTM exons. Polypyrimidine tract (PPT) and 3′-AG dinucleotide, which are critical for initial recognition of an intron, are indicated.

SMaRT TECHNOLOGY AS A VERSATILE TOOL FOR GENE REPROGRAMMING

Reprogramming of pre-mRNAs is based on exploiting naturally occurring splicing events during pre-mRNA maturation (Puttaraju *et al.*, 1999). SMaRT refers to a process wherein two distinct pre-mRNA molecules are recombined specifically in an exon-wise manner. An engineered RTM delivers a wild-type coding region of interest to replace an endogenous, mutated pre-mRNA (Figure 1). This process of *trans*-splicing is mediated by the cell's spliceosome and, although *trans*-splicing is very rare, it is a naturally occurring event that was first observed in flatworms, trypanosomes, and recently in the human estrogen receptor (Murphy *et al.*, 1986; Davis *et al.*, 1995; Flouriot *et al.*, 2002). Crucial for the production of a functional, mature, *trans*-spliced mRNA are preference of the spliceosome for the RTM over *cis*-splicing, utilization of the correct splice sites, and maintenance of the integrity of the coding region (Figure 2). Thus, an RTM must possess a number of features: (a) the wild-type coding region of the gene portion to be replaced, (b) functional splice sites, (c) spliceosomal recognition sites (e.g., polypyrimidine tract and branch point), and (d) a target recognition sequence/binding domain (BD) able to hybridize specifically to the endogenous pre-mRNA of interest. Depending on the location of the mutation to be corrected or the region to be replaced, three types of SMaRT approaches are available. The two major types are replacement of a 5′-coding cassette (5′-*trans*-splicing) and replacement of a downstream coding cassette (3′-*trans*-splicing); the third type, internal exon replacement (IER), involves exchange of one or more internal exons (Figure 3).

Recent studies have shown that the BD is of major importance for success of the *trans*-splicing process. Minor variations in sequence or target-binding position can have a major impact on the outcome of mRNA repair. Blocking of the *cis*-splice acceptor site of the target pre-mRNA seems to be crucial for the design of a BD, but rational engineering of RTMs has failed to reveal a consistent formula, and individual design for every RTM does not guarantee production of the most potent molecule. Our group has therefore modified a screening system initially suggested by Mitchell and McGarrity (2005) and Yang and Walsh (2005), which enables the identification of highly specific BDs for genes affected in epidermolysis bullosa (Wally *et al.*, 2010, 2011; Gruber *et al.*, 2011; Koller

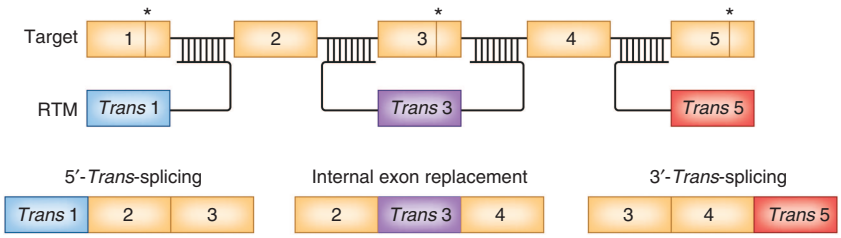

Figure 3. Spliceosome-mediated RNA *trans*-splicing can be applied to replace a 5′-, a 3′-, or an internal gene portion, referred to as 5′-*trans*-splicing, 3′-*trans*-splicing, or internal exon replacement, respectively. The mature spliced messenger RNA (mRNA) consists of the respective wild-type coding region brought in by the RNA-*trans*-splicing molecule (RTM; *trans* 1, *trans* 3, *trans* 5, respectively) and the remaining exons derived from the endogenous target pre-mRNA. * Indicates a mutation within the target coding region.

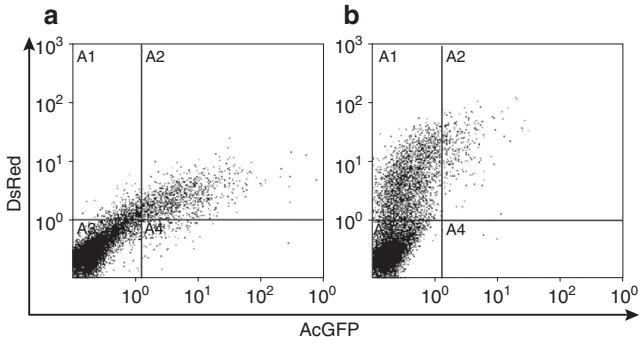

Figure 4. Two individual RNA-*trans*-splicing molecules (RTMs; RTM backbone: split reporter AcGFP and transfection reporter DsRed) with different binding domains have diverging *trans*-splicing efficiencies. (**a**) A high *trans*-splicing efficiency is characterized by a high DsRed/AcGFP ratio (sector A2) and few cells expressing DsRed only (sector A1). (**b**) A weak RTM produces cells expressing DsRed only (sector A1) and only few cells are double positive for AcGFP and DsRed (sector A2).

et al., 2010, 2011). In this fluorescence-based screen, ~10^6 of randomly generated BDs are cloned into reporter RTMs, which can be analyzed for their putative *trans*-splicing efficiency and specificity by FACS analysis. Outstanding RTMs can be isolated by FACS and characterized by sequence analysis. Analysis of numerous RTM libraries revealed the functionality of RTMs to be mainly related to length and binding position (Figure 4). However, it is also likely that the secondary structure formation, length of the targeted intron, and other unknown factors influence *trans*-splicing efficiency. As these factors are hard to predict, identification of the most-efficient BDs by large-scale FACS screening is currently the best way to obtain high-quality RTMs.

SMaRT FOR THERAPEUTIC CORRECTION OF MUTATED GENES *IN VITRO*

The functionality of SMaRT has been proven extensively in *in vitro* applications for a variety of diseases (Mitchell and McGarrity, 2005) (Table 1). Initial approaches were carried out using double-transfection systems with reporter molecules (e.g., LacZ) (Dallinger *et al.*, 2003). In such systems, a target construct expressing the desired intron and one half of a reporter gene is co-transfected with an RTM comprising a specific BD and the other half of the reporter gene. The advantage of this double-transfection system is that *trans*-splicing can easily be verified at both the mRNA and protein levels. In later experiments, mini genes were used for co-transfection studies, carrying part of the sequence of the gene of interest instead of reporter genes. This approach was chosen by Coady *et al.* (2007) for the repair of the *survival motor neuron* genes 1 and 2 (*SMN1* and *SMN2*), in which two target mini genes were cloned comprising the genomic sequence of exon 6 to exon 8. Both mini genes were co-transfected with a 3′-RTM specifically hybridizing to intron 6 of the *SMN2* gene, bringing in exon 7 and two hemagglutinin repeats for *trans*-splicing detection, into HeLa cells. Successful *trans*-splicing was detected at the mRNA and protein levels (Coady *et al.*, 2007).

In a recent study, Shababi and Lorson (2011) improved the *SMN2* *trans*-splicing efficiency by determining the intron that promotes *trans*-splicing most efficiently. A panel of RTMs targeting specific introns within the *SMN2* pre-mRNA was constructed and examined in cellular assays. The efficacy of the selected RTM targeting intron 3 was further improved by introducing an antisense cassette that blocks the splice acceptor site of the downstream exon.

In addition, for the correction of *tau* mis-splicing that underlies frontotemporal dementia with parkinsonism, double transfection of mini genes with appropriate RTMs was carried out. Rodriguez-Martin *et al.* (2005, 2009) showed both directed exon 10 inclusion and exclusion at the mRNA level (Rodriguez-Martin *et al.*, 2005, 2009). Another type of double transfection was investigated by Song *et al.* (2009) for the 4.44-kb cystic fibrosis transmembrane conductance regulator (*CFTR*). IB3-1 cells, which are compound heterozygous for the *CFTR* hotspot mutation delF508 and a nonsense mutation W1282X, were transduced with two RTMs delivering the 5′- and the 3′-halves of *CFTR*, the 5′- and 3′-donor and acceptor splice sites, respectively, and a hybridization domain. *Trans*-splicing between the transcripts derived from both vectors resulted in mature, full-length *CFTR* mRNA (Song *et al.*, 2009).

As mentioned above, IER (Figure 3) is a combination of 5′- and 3′-*trans*-splicing that is used to replace a central portion of a given transcript. Whereas theoretically this is an elegant approach, its practical application has been hampered by low efficiency. The feasibility of IER was demonstrated in a co-transfection system (Lorain *et al.*, 2010). Here, a mini gene–encoding part of the *mdx* dystrophin gene encompassing a mutation in exon 23 and a specific RTM resulted in correction of

Table 1. SMaRT applied to gene correction *in vitro*

Gene	Disease	RTM	Author, year
Collagen VII (COL7A1)	Recessive dystrophic epidermolysis bullosa	3′	Murauer *et al.*, 2011
Keratin 14 (K14)	Epidermolysis bullosa simplex, Dowling Meara	5′	Wally *et al.*, 2010
Dystrophin (MDX)	Duchenne muscular dystrophy	IER	Lorain *et al.*, 2010
Cystic fibrosis transmembrane conductance regulator (CFTR)	Cystic fibrosis	5′+3′	Song *et al.*, 2009
Microtubule associated protein (MAPT)	Frontotemporal dementia with parkinsonism	3′	Rodriguez-Martin *et al.*, 2009
Plectin (PLEC)	Epidermolysis bullosa simplex with muscular dystrophy	5′	Wally *et al.*, 2008
DNA-PKcs	Severe combined immune deficiency	3′	Zayed *et al.*, 2007
Survival motor neuron (SMN2)	Spinal muscular atrophy	3′	Coady *et al.* 2007
ß-globin	Sickle cell anemia, ß-thalassemia	5′	Kierlin-Duncan and Sullenger, 2007
Cystic fibrosis transmembrane conductance regulator (CFTR)	Cystic fibrosis	3′	Liu *et al.*, 2005
Microtubule associated protein (MAPT)	Frontotemporal dementia with parkinsonism	3′	Rodriguez-Martin *et al.*, 2005

Abbreviations: RTM, RNA-*trans*-splicing molecule; SMaRT, spliceosome-mediated RNA *trans*-splicing.
Summary of published studies from 2005 to 2011.

exon 23 on the target mRNA with an efficiency of up to 45%. Recently, Koller *et al.* (2011) expanded the functionality of IER in a double-transfection approach using a novel split GFP reporter system. A target molecule was designed consisting of *COL17A1* exon 52 flanked by introns 51 and 52, as well as the 5′- and 3′-thirds of the split *GFP* gene. *Trans*-splicing with an RTM that included BDs for both introns and all three split parts of the *GFP* resulted in accurate substitution of exon 52 and subsequent restoration of a full-length GFP mRNA and protein, confirmed by the appearance of green fluorescence (Koller *et al.*, 2011).

Trans-splicing to endogenous pre-mRNA is the only form of SMaRT in terms of clinical utility. For that, 5′-*trans*-splicing and 3′-*trans*-splicing approaches have been published (Wally *et al.*, 2008, 2010; Mayr *et al.*, 2011; Murauer *et al.*, 2011). Wally *et al.* (2008, 2010) corrected the 5′-portion of *PLEC* in epidermolysis bullosa simplex fibroblasts and exons 1 to 7 of the *keratin 14* gene in patient keratinocytes. Successful *trans*-splicing was detected at the RNA and protein levels, as well as by functional studies that included scratch and invasion assays (Wally *et al.*, 2008, 2010). For recessive dystrophic epidermolysis bullosa (RDEB), a downstream 3.3-kb fragment of *COL7A1* was replaced by 3′-*trans*-splicing, resulting in restored protein expression and integrity of the basement membrane zone of skin equivalents made from corrected patient keratinocytes (Murauer *et al.*, 2011). Initial results are also available for the 5′-portion of the *COL7A1* gene (Mayr *et al.*, 2011). Endogenous *trans*-splicing was also shown for *CFTR*, the ß-globin gene, and the DNA protein kinase gene (Liu *et al.*, 2005; Kierlin-Duncan and Sullenger, 2007; Zayed *et al.*, 2007). Whereas 5′-*trans*-splicing was applied to the ß-globin gene, the other two mRNAs were corrected by 3′-*trans*-splicing. Major differences lie in the vector-delivery strategy. Although for most approaches viral vectors are used, Zayed *et al.* (2007) integrated the RTM using a Sleeping Beauty transposon, proposing that this method should afford increased safety and reduced immunogenicity on account of its lower efficiency.

SMaRT FOR THERAPEUTIC CORRECTION OF MUTATED GENES *IN VIVO*

The first SMaRT approach applied to a mouse model was carried out by Chao *et al.* (2003) for the bleeding disorder hemophilia A. A 3′-RTM was designed to repair mutant factor VIII mRNA in F8-knockout mice exhibiting a hemophilia A phenotype. SMaRT-treated mice expressed functional factor VIII proteins and were able to clot and survive a tail-vein cut.

Later, Tahara *et al.* (2004) corrected hyper-IgM X-linked immunodeficiency by 3′-*trans*-splicing repair of the CD40 ligand (CD40L) in a CD40-knockout mouse model. In this *ex vivo* gene therapy approach, bone marrow cells from mice lacking CD40L were corrected with an RTM-expressing wild-type CD40L and transplanted into syngeneic CD40L-knockout mice. CD40L mRNA-*trans*-splicing repair led to functional correction of the genetic defect in treated mice. It is noteworthy that in earlier experiments the transduction of $CD40L^{-/-}$ mice with thymic or bone marrow cells that expressed CD40L cDNA from an integrated retrovirus produced T-lymphoproliferative disorders in 12 of 19 cases (Brown *et al.*, 1998).

Recently, Coady and Lorson (2010) performed 3′-*trans*-splicing in two different mouse models of spinal muscular atrophy (SMA). The autosomal recessive forms of SMA, resulting from homozygous loss of the *SMN1* gene, are the most commonly inherited causes of infant death. *SMN2* is a highly homologous gene that differs from *SMN1* by a few nucleotide changes, one of which leads to alternative splicing and expression of a truncated, nonfunctional protein lacking amino acids encoded by exon 7. Coady *et al.* (2007) used *SMN2* as a target for 3′-*trans*-splicing, aiming to increase the level of functional *SMN2* protein. An RTM specifically hybridizing to intron 6 was engineered,

Table 2. SMaRT applied to gene correction *in vivo*

Gene	Disease	RTM	Author, year
Survival motor neuron (*SMN2*)	Spinal muscular atrophy	3′	Coady and Lorson, 2010
Survival motor neuron (*SMN2*)	Spinal muscular atrophy	3′	Shababi *et al.*, 2011
Collagen VII (*COL7A1*)	Dystrophic epidermolysis bullosa	3′	Murauer *et al.*, 2010 (abstr.)
Survival motor neuron (*SMN2*)	Spinal muscular atrophy	3′	Coady *et al.*, 2008
CD40 Ligand (*CD40L*)	Hyper-IgM X-linked immunodeficiency	3′	Tahara *et al.*, 2004
Factor VIII (*F8*)	Hemophilia A	3′	Chao *et al.*, 2003

Abbreviations: RTM, RNA-*trans*-splicing molecule; SMaRT, spliceosome-mediated RNA *trans*-splicing.
Summary of published studies from 2005 to 2011.

bringing in wild-type exon 7. To increase the efficiency, co-application of an antisense oligonucleotide was performed to inhibit undesired splicing to the *cis*-splice site (Horne and Young, 2009). The first *in vivo* application was achieved in a SMA murine model referred to as SMNΔ7, expressing the human *SMN2* gene and the human *SMNΔ7* cDNA (Coady *et al.*, 2008). The second murine model was a more severe SMA mouse model lacking murine *SMN* but expressing two genomic copies of human *SMN2* ($SMN^{-/-}$/$hSMN2^{+/+}$) (Coady and Lorson, 2010; Shababi *et al.*, 2011). Increased life span (average increase 3 days) of mice was seen after a single injection of a vector carrying the *trans*-splicing RNA sequences, as well as an antisense oligonucleotide into the intracerebral–ventricular space. Furthermore, successful *trans*-splicing was confirmed by reverse transcriptase–PCR and western blotting (Table 2).

In a mouse model of RDEB, it was demonstrated that 3′-*trans*-splicing of the *COL7A1* gene generates stable expression of human type VII collagen *in vivo* (Murauer *et al.*, 2010). Patches of skin equivalents cultured from corrected RDEB keratinocytes were grafted onto immunodeficient mice. Histological and immunohistological analysis of 5-week-old specimens of grafted tissue showed no blistering and strong labeling of human type VII collagen between the dermis and epidermis. Localization of type VII collagen was restricted to the basement membrane, with no expression in the suprabasal cell layers, as expected for proper expression.

SMaRT BEYOND GENE CORRECTION

Suicide gene therapy

Delivery of RTMs encoding sequences of endotoxins or exotoxins can be used as a strategy for cancer therapy. Nakayama *et al.* (2005) developed a segmental *trans*-splicing approach in which 5′-donor and 3′-acceptor segments encoding an intracellular toxin were co-delivered into cancer cells using viral vectors (Nakayama *et al.*, 2005). Upon accurate *trans*-splicing, a functional toxin was produced, inducing apoptotic cell death. This method should prevent the toxicity of such vectors for producer cell lines, which has limited the use of toxins for cancer therapy.

Recently, Gruber *et al.* (2011) used SMaRT technology in a suicide therapy approach for squamous cell carcinoma (SCC) occurring in RDEB patients (Gruber *et al*, 2011). They showed that endogenous *trans*-splicing of an exotoxin into a tumor marker gene could induce tumor-specific cell death. In this case, a 3′-RTM encoding a truncated version of the exotoxin streptolysin O was used to specifically target MMP-9, a marker gene that is highly upregulated in cultured RDEB–SCC cells. *Trans*-splicing of streptolysin O into the endogenous *MMP-9* pre-mRNA led to expression of functional streptolysin O and preferential death of RDEB–SCC cells but not of noncancerous RDEB cells, demonstrating the high specificity of the *trans*-splicing reaction.

Production of therapeutic proteins

There have been attempts to utilize SMaRT technology for the *in vitro* production of therapeutic proteins such as antibody fusion proteins (Schlesinger *et al.*, 2003; Iwasaki *et al.*, 2009). Some early-stage studies are currently evaluating the feasibility of SMaRT to generate antibodies endogenously expressed in the human body, replacing the application of commercially produced therapeutic antibodies (http://www.virxsys.com/pages/technology-platforms/smart-rna-platform.php). For this approach, RTMs encoding a therapeutic antibody will be constructed to reprogram a specific gene transcript in the targeted cell, which will then express and secrete the desired antibody into the plasma. Proof of principle has already been demonstrated by Wang *et al.* (2009) who cloned separately the full-length coding regions for factor VIII (hemophilia A), apo-AI (a major component of the high-density lipoprotein), and a single-chain antibody specific for human papillomavirus type-16 E7 oncoprotein (HPV16-E7), each without the initiation codon, into a 3′-RTM, which contained a BD hybridizing to intron 1 of the albumin gene. In each case, the resulting *trans*-spliced mRNA was a chimeric molecule composed of the 5′-UTR and exon 1 of the albumin gene and the respective coding sequence of the therapeutic protein. All three approaches were tested in mouse models: HPV16-E7 single-chain antibody and apo-AI were applied to C57BL/6 mice and factor VIII to hemophilia A mice by hydrodynamic tail-vein injection. Successful *trans*-splicing and functionally mature proteins were verified in mouse serum (factor VIII, apo-AI, and HPV16-E7) and liver (factor VIII and apo-AI).

Molecular imaging

Molecular imaging of gene expression is another application of SMaRT, in which any reporter gene can be *trans*-spliced into any pre-mRNA of interest. Current limitations for direct molecular imaging include the difficulties of

Table 3. SMaRT applications beyond gene therapy

Application	RTM	Animal model	Author, year
Suicide therapy of RDEB–SCC	3′		Gruber *et al.*, 2011
Antibody production	3′		Iwasaki *et al.*, 2009
Therpeutic protein production by splicing into albumin	3′	+	Wang *et al.*, 2009
Luciferase-based imaging of gene expression	3′	+	Walls *et al.*, 2008
Segmental *trans*-splicing for cancer suicide therapy	5′ + 3′		Nakayama *et al.*, 2005

Abbreviations: RDEB, recessive dystrophic epidermolysis bullosa; RTM, RNA-*trans*-splicing molecule; SCC, squamous cell carcinoma; SMaRT, spliceosome-mediated RNA *trans*-splicing.
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design and delivery of imaging probes. SMaRT offers the advantages of rapid development of imaging probes on the basis of sequence information only, and simplified delivery using existing gene-delivery systems. As the copy number of RNA transcripts reflects the level of gene expression, SMaRT real-time molecular imaging is suitable for both preclinical research and human diagnostics.

Reporter-gene imaging of RNA by using SMaRT has been achieved in both cells and living animals (Bhaumik *et al.*, 2004; Walls *et al.*, 2008). Whereas Bhaumik *et al.* (2004) provided proof of principle of SMaRT-mediated imaging of gene expression, Walls *et al.* (2008) expanded this concept to a general strategy for real-time RNA imaging in living animals. They developed a luciferase-based model system of an artificial target gene and a suitable RTM to image gene expression at the pre-mRNA level *in vitro* and in nude mice. Specific *trans*-splicing was observed in living mice, demonstrating the feasibility of SMaRT for quantification of gene expression at the RNA level *in vivo* (Table 3).

SMaRT LIMITATIONS

Limitations of SMaRT mainly concern the potential for nonspecific *trans*-splicing to other endogenous pre-mRNAs (Kikumori *et al.*, 2001). Potential sequence homologies in the binding region and the presence of a strong 5′- or 3′-splice site on the RTM may be the main reasons for nonspecific *trans*-splicing events. The consequences of such unwanted side reactions are hard to predict and depend on the gene function of the *trans*-spliced transcript. However, screening of an optimal BD and mapping of the BD sequence against the human genome to exclude sequence homologies should widely reduce nonspecific events even though their complete elimination cannot be guaranteed. Up until now, no unwanted side effects have been reported in cell culture, but they have yet to be studied in animal models.

Trans-splicing efficiencies have reached up to 99% in reporter-based double-transfection systems, but much lower levels are typical in endogenous settings. However, these lower levels can be sufficient to revert phenotypes, especially in autosomal recessive diseases, but also in dominant diseases where a concomitant increase in the level of expression of wild-type alleles and knockdown of mutated alleles multiplies the effect of SMaRT. In addition, correction levels of mRNA hardly ever have to be 100% for phenotypic correction. For example, for keratin 14, Werner *et al.* (2004) showed that dominance is limited and depends on the ratio of wild-type and mutated alleles, which was also shown by Cao *et al.* (2001) in an inducible mouse model. Moreover, similar results were obtained for type VII collagen, as overexpression of the wild-type *COL7A1* gene corrected a dominant phenotype (Fritsch *et al.*, 2009).

Vector transfer remains a crucial limitation of any gene therapy approach. It is important to transfer the RNA to the nucleus, which is the reason why mainly viral vectors are used. However, the ever-present problem when using any type of lentiviral or retroviral vector in gene therapy is the unpredictability of the site of integration of the transgene in the cell's genome, a fact that is independent of the SMaRT technology. As an alternative to viral transfer, the transposon Sleeping Beauty was used for RTM delivery and stable integration into the cell's genome (Zayed *et al.*, 2007). However, current limitations of transposon technology for gene therapeutic applications include the lack of long-term therapeutic transgene expression due to inefficiency of transgene delivery.

SUMMARY

Since previous reviews were published in 2005 (Mitchell and McGarrity, 2005; Yang and Walsh, 2005), SMaRT has advanced from double-transfection approaches (Puttaraju *et al.*, 2001; Dallinger *et al.*, 2003; Mansfield *et al.*, 2003) to endogenous application, which is now well established. SMaRT was shown to be potent enough to work in an endogenous setting, resulting in the reprogramming of a considerable number of mRNA alleles by 3′-*trans*-splicing and 5′-*trans*-splicing. First mouse models (Chao *et al.*, 2003; Coady and Lorson, 2010; Murauer *et al.*, 2010) confirmed the efficacy of SMaRT *in vivo*, which is a major step toward clinical application. Furthermore, SMaRT technology has broadened beyond RNA therapy for genetic disorders. For example, reporter-gene imaging of pre-mRNA molecules by *trans*-splicing was achieved in animal experiments by Walls *et al.* (2008), illustrating the potential of the *trans*-splicing technique for real-time monitoring of expression of any given gene at the pre-mRNA level in living subjects.

CONFLICT OF INTEREST

A patent on ''Improved pre-mRNA *trans*-splicing molecule (RTM) molecules and their uses'' is pending.

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